

HEPP, A NOVEL GENE WITH A ROLE IN HEMATOPOIETIC AND NEURAL DEVELOPMENT

[0001] This application claims priority to U.S. application no. 60/268,923, filed February 16, 2001, which is incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The invention relates to a novel conserved gene and protein product, designated *Hepp* (hematopoietic progenitor protein, that has a role in mammalian hematopoiesis and neural function.

BACKGROUND INFORMATION

[0003] The life-long maintenance and regenerative capacity of the hematopoietic system depends upon self-renewal and differentiation of pluripotent hematopoietic stem cells (HSC). The HSC give rise to all mature blood cell types by differentiating through intermediate progenitor cells that undergo lineage commitment and subsequent development along a single pathway (1–5). During the last two decades a highly complex regulatory network of molecular mechanisms, necessary to control lineage commitment and differentiation of blood cells has been identified, including growth factors and receptors, cell-cell interaction molecules, signal transduction molecules and transcription factors (6 –15). Due to distinct functional features of HSC, progenitors and mature blood cells, it is reasonable to assume that these properties are regulated at least in part by molecules that are preferentially expressed at particular stages of blood cell development. One approach to identify molecules that are important for self-renewal and lineage commitment of HSC and progenitors is to focus on rare populations of cells that are enriched for HSC and progenitors. Construction of HSC and progenitor cell-specific subtracted cDNA libraries, coupled with cDNA sequencing and microarray-based studies of gene expression patterns, will be necessary to molecularly define self-renewal, functional pluripotency and lineage commitment of HSC and progenitors and to elucidate the extraordinary developmental plasticity of HSC (16 –19). Using subtracted cDNA libraries and cDNA microarray approach Phillips *et al.* (17) have recently reported results of a genome-wide gene

10076059.021502

expression analysis in mouse fetal liver HSC and progenitors. The complete data in the form of a database represent the first step in elucidating the molecular phenotype of hematopoietic stem cells and progenitors.

[0004] Elucidation of the differential gene expression during differentiation of hematopoietic stem cell and progenitors should have far reaching implications for ex vivo manipulation of HSC, clinical bone marrow transplantation and gene therapy of hematological disorders.

[0005] To identify novel molecules involved in intrinsic regulation of HSC and progenitor cell lineage commitment and differentiation we have generated full-length and subtracted cDNA libraries from mouse adult bone marrow cell populations enriched for HSC (Lin⁻Sca-1⁺ cells) and progenitors (Lin⁻Sca-1⁻ cells) (19). Phenotypically and functionally defined population of primitive Lin⁻Sca-1⁺ cells comprises 0.1-0.2% of bone marrow cells and contains virtually all HSC and primitive progenitors, whereas more differentiated Lin⁻Sca-1⁻ cells contain committed progenitors but lack HSC. Here we describe cloning and characterization of a novel gene, *Hepp*, that is expressed preferentially in mouse fetal and adult hematopoietic progenitors and mature blood cells.

[0006] Certain aspects of the present invention have been disclosed in Abdullah et al. (19).

SUMMARY OF THE INVENTION

[0007] Through differential screening of mouse hematopoietic stem cells (HSC) and progenitor subtracted cDNA libraries, we have identified a progenitor cell-specific transcript that represents a novel conserved gene, designated *Hepp* (hematopoietic progenitor protein). Mouse and human *Hepp* genes encode proteins of 267 and 241 amino acids with no detectable known functional domains or motifs. The mouse gene and corresponding protein are set forth in SEQ ID NO:1 and SEQ ID NO:2, respectively. The human gene and corresponding protein are set forth in SEQ ID NO:3 and SEQ ID NO:4, respectively. During embryonic hematopoiesis *Hepp* is not expressed in mouse fetal liver HSC (Sca-1⁺ *c-kit*⁺ AA4.1⁺ Lin⁻ cells), but is abundantly transcribed in populations of hemotopoietic progenitors (AA4.1⁻). In adult mice, *Hepp* is not

transcribed in highly enriched populations of bone marrow HSC ($\text{Rh}^{-}\text{123}^{\text{low}}\text{Sca-1}^{+}\text{c-kit}^{+}\text{Lin}^{-}$ cells), but its expression is unregulated as more heterogeneous population of bone marrow HSC ($\text{Lin}^{-}\text{Sca-1}^{+}$ cells) differentiates into progenitors ($\text{Lin}^{-}\text{Sca-1}^{-}$ cells) and more mature lymphoid and myeloid cell types. The human gene was localized to chromosome 14q32, a region with frequent chromosome aberrations associated with multiple cases of acute myeloid leukemia, chronic lymphoproliferative disorder, acute lymphoblastic leukemia, non-Hodgkin's lymphoma, and myelodysplastic syndrome, for which the genes involved are unknown. Evolutionary conservation and differential expression in fetal and adult HSC and progenitors suggest that *Hepp* gene could play an important role in HSC/progenitor cell lineage commitment and differentiation, and could be involved in etiology of hematological malignancies.

[0008] The gene and associated protein should be useful in a variety of contexts, for example, as reagents for differential identification of the tissue(s) or cell type(s) present in a biological sample and for diagnosis of diseases and conditions which include, but are not limited to, neural disorders and malignancies, particularly malignancies of the blood. Polypeptides of the invention and antibodies directed to these polypeptides are expected to be useful in providing immunological probes for differential identification of the tissue(s) or cell type(s), by means familiar to persons of skill in the art. For a number of disorders of neural and hematological tissues or cells, particularly of the nervous system and blood, expression of the *Hepp* gene at significantly higher or lower levels may be routinely detected in certain tissues or cell types relative to the standard gene expression level, i.e., the expression level in healthy tissue or bodily fluid from an individual not having the disorder.

[0009] The tissue distribution of *Hepp* gene expression, and the characteristics of the *Hepp* knock-out mouse described herein, indicate that polynucleotides and polypeptides corresponding to this gene are useful for the detection, treatment, and/or prevention of neurodegenerative disease states, such as, for example, amyotrophic lateral sclerosis, and hematological disorders, particularly neoplasms of the blood such as acute myelomonocytic leukemia, lymphoblastic lymphoma, chronic lymphocytic leukemia, acute lymphoblastic leukemia, multiple myeloma, B-prolymphocytic leukemia, plasma cell leukemia, adult T-cell lymphoma/leukemia, diffuse large B-cell lymphoma, nodal marginal zone B-cell lymphoma, Burkitt's lymphoma, follicular

lymphoma, hairy cell leukemia, mantle cell lymphoma, splenic marginal zone B-cell lymphoma, and T-prolymphocytic leukemia.

[0010] The terms "nucleic acid" "oligonucleotide", and "polynucleotide" are intended to include RNA, DNA, or RNA/DNA hybrid sequences of more than one nucleotide in either single chain or duplex form, and are used interchangeably herein.

[0011] The terms "peptide", "polypeptide" and "protein", as used herein, refer to a sequence of naturally occurring amino acids, more particularly to a translated amino acid sequence generated from a polynucleotide of the invention. The proteins of the invention may in some instances have undergone postranslational modification. In general, "peptide" refers to a sequence of less than 10 residues, "polypeptide" refers to a sequence of 10 or more amino acid residues and as used herein is intended to encompass proteins as well.

[0012] The terms "complementary" or "complement thereof", as used herein, refer to sequences of polynucleotides which are capable of forming Watson & Crick base pairing with another specified polynucleotide throughout the entirety of the complementary region. This term is applied to pairs of polynucleotides based solely upon their sequences and does not refer to any specific conditions under which the two polynucleotides would actually bind.

[0013] For the purposes of this invention, when referring to nucleic acid and polypeptide sequences, percent similarity and percent identity are calculated according to the methods of CLUSTAL W (32).

[0014] As used herein, the term "isolated" refers to material removed from its original environment (e.g., for naturally occurring substances, removed from their natural environment). Such material could be part of a vector or a composition of matter, or could be contained within a cell, if said vector, composition or cell is not the original environment of the material.

[0015] As used herein, the term “transgenic animal” is an animal containing a defined change to its germ line, wherein the change is not ordinarily found in the wild-type animal and can be passed on to the animal's progeny. The change to the animal's germ line can be an insertion, a substitution, or a deletion. In a broad sense, the term “transgenic” encompasses organisms where a gene has been eliminated or disrupted so as to result in the elimination of a phenotype associated with the disrupted gene (“knock-out (KO) animals”). The term “transgenic” also encompasses organisms containing modifications to their existing genes and organisms modified to contain exogenous genes introduced into their germ line.

[0016] It is one object of the invention to provide an isolated nucleic acid comprising a sequence that is at least 70% identical to SEQ ID NO:1 or SEQ ID NO:3, or a sequence that is complementary thereto. Preferably the sequence is at least 77% identical, more preferably 80% identical, and even more preferably 85%, 90%, 95%, 98%, 99% or 100% identical to SEQ ID NO:1 or SEQ ID NO:3.

[0017] The invention also provides an isolated nucleic acid comprising a sequence that is at least 70% identical to a fragment of SEQ ID NO:1 or SEQ ID NO:3, the fragment representing at least 50 contiguous bases, preferably 100 contiguous bases and most preferably 150 contiguous bases of SEQ ID NO:1 or SEQ ID NO:3. Preferably the sequence is at least 77% identical, more preferably 80% identical, and even more preferably 85%, 90%, 95%, or 100% identical to said contiguous bases.

[0018] The nucleic acids of the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art, and may be cloned using techniques known in the art. In this regard, the invention also includes a vector comprising the nucleic acid of the invention, and a host cell comprising the nucleic acid of the invention.

[0019] The invention also provides a polypeptide or protein comprising an amino acid sequence that is at least 70% identical to SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7. Preferably the sequence is at least 75% identical, more preferably

80% identical, and even more preferably 85%, 90%, 95%, 98%, 99% or 100% identical to one of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7. In an alternate embodiment, the amino acid sequence is at least 60%, preferably 70%, 75%, 80%, and more preferably 85%, 90%, 95%, 98%, 99% or 100% similar to SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7.

[0020] The invention also provides an isolated polypeptide or protein comprising a sequence that is at least 70% identical to a fragment of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7, the fragment representing at least 10 contiguous amino acid residues, preferably 20 contiguous amino acid residues and most preferably 50 contiguous residues of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7. Preferably the sequence is at least 75% identical, more preferably 80% identical, and even more preferably 85%, 90%, 95%, 98%, 99% or 100% identical to said contiguous residues.

[0021] The polypeptide of the present invention may be produced by conventional methods of chemical synthesis or by recombinant DNA techniques. For example, a host microorganism may be transformed with a DNA fragment encoding the polypeptide and the polypeptide harvested from the culture. The host organism may be, for example, a bacterium, a yeast or a mammalian cell, whereby the DNA fragment in question is integrated in the genome of the host organism or inserted into a suitable expression vector capable of replicating in the host organism. The DNA fragment is placed under the control of regions containing suitable transcription and translation signals. Methods for obtaining polypeptides by these means are familiar to persons skilled in the art.

[0022] The invention also provides a non-human mutant vertebrate, or "knock-out (KO) animal" in which the *Hepp* gene has been impaired at one or both loci in somatic and germ cells. A "knock-out animal" is an animal in which selected genes have been mutated to prevent expression of functional protein products. In this regard, the invention provides a non-human mutant vertebrate, in which all or some of the germ and somatic cells contain a mutation in at least one *Hepp* locus, which mutation is introduced into the vertebrate, or an ancestor of the vertebrate, at an embryonic stage. The term "vertebrate" encompasses mammals, birds, reptiles,

amphibians, and fishes that possess a *Hepp* gene or equivalent. Preferably the vertebrate is a non-human mammal, most preferably a mouse, rat, or rabbit.

[0023] In one preferred embodiment, the mutation produces a phenotype in a mammal characterized by perturbed hematopoiesis consisting of bone marrow cytopenia, overproduction and/or accumulation of hematopoietic progenitors, and splenomegaly with follicular hyperplasia. In an especially preferred embodiment, the vertebrate is a mouse that is heterozygous or homozygous for HEPP⁻, a knock-out gene that results in a reduction or absence of functional HEPP protein. Such mice can be obtained by treating mouse embryos with ES cell clone KST303. Other means of producing mutant animals, such as knock-in techniques, are familiar to those of skill in the art.

[0024] The invention also provides a means of producing a KO mammal, in particular a mouse, that is heterozygous or homozygous for a defective *Hepp* gene (e.g. *Hepp*⁻). In one method of producing the transgenic animals, transformed ES cells containing a disrupted *Hepp* gene having undergone homologous recombination, are introduced into a normal blastocyst. The blastocyst is then transferred into the uterus of a pseudo-pregnant female for gestation and delivery. Resulting heterozygous mutant animals are then bred to obtain homozygous mutant animals. Other means of producing KO animals are familiar to those of skill in the art. Examples are disclosed in U.S. Pat. No. 6,015,676 (Lin et al.) and Gene Knockout Protocols. In: Methods in Molecular Biology, vol. 158, 2001. Edited by: M.J. Tymms and I. Kola. Humana Press, Totowa, New Jersey, incorporated herein by reference.

[0025] The mutant vertebrate of the invention may be one in which all of the germ and somatic cells contain the mutation, i.e., the vertebrate is either a heterozygote or a homozygote for the mutation. The vertebrate may be one wherein both of the *Hepp* alleles in all of the germ and somatic cells contain the mutation, i.e., the vertebrate is a homozygote for the mutation. Alternatively, the vertebrate may be a chimera (an animal in which only some of the germ and somatic cells contain the mutation).

[0026] The mutant vertebrate of the invention should be useful, *inter alia*, in screening drugs for the treatment of neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS) and testing of novel hematopoietic cytokines/growth factors for mobilization and differentiation of stem and progenitor cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027] Fig. 1A. Complementary DNA sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) for the mouse *Hepp* gene. The 5' in-frame stop codon found upstream of the start codon is underlined in the nucleotide sequence. The stop codon is indicated by an asterisk. The polyadenylation signal-like sequence is underlined in bold. The nucleotide sequence data reported here appear in the GenBank nucleotide sequence databases under Accession No. AF322238.

[0028] Fig. 1B. Complementary DNA sequence (SEQ ID NO:3) and deduced amino acid sequence (SEQ ID NO:4) for the human *HEPP* gene. The 5' in-frame stop codon found upstream of the start codon is underlined in the nucleotide sequence. The stop codon is indicated by an asterisk. The polyadenylation signal-like sequence is underlined in bold. The nucleotide sequence data reported here appear in the GenBank nucleotide sequence databases under Accession No. 322239.

[0029] Fig. 2. ClustalW amino acid sequence alignment of the mouse (SEQ ID NO: 2) and human (SEQ ID NO:4) HEPP proteins.

[0030] Fig. 3. Amino acid sequence alignment of the N terminal portion of zebrafish (SEQ ID NO:5), mouse (SEQ ID NO:6) and human (SEQ ID NO:7) HEPP proteins.

[0031] Fig. 4. Northern analysis of *Hepp* expression in adult mouse tissues. (a) *Hepp* is transcribed at a very low level in heart, lung, spleen, and thymus and at a higher level in muscle. (b) Hybridization with actin probe as a positive control.

[0032] Fig 5. Semiquantitative duplex PCR and RT-PCR expression analysis of *Hepp* and HPRT (control) in mouse fetal liver and adult bone marrow HSC and progenitor cell populations.

[0033] Fig. 6. Semiquantitative duplex RT-PCR expression analysis of *Hepp* and HPRT in various hematopoietic cell lines demonstrates that mouse *Hepp* is ubiquitously expressed in different stages of lymphoid and myeloid cell development.

[0034] Fig. 7. Chromosomal localization of *Hepp* and hematological malignancies associated with rearrangements of the band q32 on chromosome 14.

[0035] Fig 8. *Hepp* is ubiquitously expressed in neural stem cells and progenitors and differentiated neural cell types.

[0036] Fig. 9. Expression pattern of mouse *Hepp* in central and peripheral nervous system.

[0037] Fig. 10. Genotyping of the progeny from the breeding of heterozygous *Hepp*^{+/-} mice.

[0038] Fig. 11. Significantly reduced number of BM cells in femurs and tibias of *Hepp*^{+/-} mice.

[0039] Fig. 12. Decreased content of B cells, granulocytes, macrophages and erythroblasts in the BM of *Hepp*^{+/-} mice (flow cytometry analysis).

[0040] Fig. 13. Increased content of BM cell populations containing progenitors and HSC in *Hepp*^{+/-} mice as analyzed by flow cytometry.

[0041] Fig. 14. Increased content of myelo-erythroid and lymphoid progenitors in the BM of *Hepp*^{+/-} mice (colony-forming assays)

[0042] Fig. 15A-B. Splenomegaly in *Hepp*^{+/-} mice.

Spleens of *Hepp*^{+/+} (15A) and *Hepp*^{+/-} (15B) mice

[0043] Fig. 15C. Significantly increased number of splenocytes in *Hepp*^{+/-} mice.

[0044] Fig. 16. Increased content of B220⁺ cells in the spleen of *Hepp*^{+/-} mice (flow cytometry analysis).

[0045] Fig. 17. Decreased content of CD8⁺ T cells in the spleen of *Hepp*^{+/-} mice (flow cytometry analysis).

[0046] Fig. 18. Progressive neurodegenerative disease in affected *Hepp*^{+/-} mice.

DETAILED DESCRIPTION OF THE INVENTION

MATERIALS AND METHODS

Fluorescence-Activated Sorting of Mouse Hematopoietic Stem and Progenitor Cells.

[0047] Phenotypically and functionally defined populations of primitive Lin⁻Sca-1⁺ cells (comprising 0.1–0.2% of bone marrow cells and containing virtually all HSC and primitive progenitors) and more differentiated Lin⁻Sca-1⁻ cells (containing committed progenitors but lacking HSC) (20–23) were isolated from the bone marrow of 6- to 8-week-old C57BL/6J mice (Taconic, Germantown, NY). Cell sorting was conducted as described previously (21), using the FACStar Vantage flow cytometer (Becton–Dickinson Immunocytometry Systems, San Jose, CA).

Library Construction and Subtractive Hybridization

[0048] Poly(A)⁺ RNA (0.5 μg) was isolated from sorted Lin⁻Sca-1⁺ and Lin⁻Sca-1⁻ cells using Micro-FastTrack mRNA isolation procedure (Invitrogen). Full-length Lin⁻Sca-1⁺ and Lin⁻Sca-1⁻ cell-specific cDNA libraries were constructed in λZAPII vector using CapFinder cDNA library construction method, according to manufacturer's protocol (Clontech, Palo Alto, CA, USA). Lin⁻

Sca-1⁺ (titer 4.8 X 10¹⁰ pfu/ml) and Lin⁻Sca-1⁻ (titer 5.6 X 10¹⁰ pfu/ml) cell-specific libraries were arrayed (2 X 10⁶ clones) into a 96-well format for efficient PCR-based screening (24). Lin⁻Sca-1⁺ and Lin⁻Sca-1⁻ cell-specific subtracted cDNA libraries were constructed by suppression subtractive hybridization (25, 26) using a PCR-Select kit (Clontech). Double-stranded cDNAs were synthesized from mouse Lin⁻Sca-1⁺ and Lin⁻Sca-1⁻ bone marrow cell poly(A)⁺ RNA, digested with *Rsa*I, and used as both tester and driver in reciprocal subtractive hybridization. After two rounds of hybridization portions of reactions were subjected to two rounds of PCR to selectively amplify differentially expressed cDNAs, which were cloned into pGEM-T vector (Promega, Madison, WI). Individual clones from subtracted cDNA libraries were arrayed as dot blots in a 96-well format and hybridized with labeled probes derived from tester and driver cDNAs (19). Confirmed differentially expressed cDNA clones were sequenced and analyzed using computer-assisted search of GenBank/EMBL and UniGene databases (www.ncbi.nlm.nih.gov/UniGene/index.html).

Cloning and Sequence Analysis of Hepp cDNA

[0049] Mouse cDNA for *Hepp* was isolated by PCRbased screening of arrayed full-length Lin2Sca-12 cell-specific cDNA library (24). The longest isolated clones were sequenced and derived *Hepp* cDNA was analyzed using the nonredundant and EST division of the GenBank database, UniGene database, and SMART (Simple Modular Architecture Research Tool, <http://smart.embl-heidelberg.de/>). Proteome WormPD database (<http://www.proteome.com/databases>) and DRES (*Drosophila* Related Expressed Sequences) Search Engine (<http://hercules.tigem.it/DRES/dres.html>) (27) were used to identify *Caenorhabditis elegans* and *Drosophila* orthologs of *Hepp*.

Expression Analysis

[0050] Mouse multiple tissue Northern blot was purchased from OriGene Technologies Inc. (Rockville,MD). *In vitro* transcribed partial *Hepp* cDNA was labeled with North2South HRP Direct labeling kit (PIERCE, Rockford, IL) and used as a nonradioactive probe. Blot was prehybridized (30 min) and hybridized (1 h) at 55°C, washed according to manufacturer's instructions, and exposed to X-ray film (Kodak) using Du Pont intensifying screens. Hybridization with non-radioactively labeled actin probe was used as a positive control.

[0051] Expression in mouse fetal and adult HSC and progenitor cell populations was analyzed by (a) semiquantitative PCR screening of cDNA libraries from fetal liver HSC (Sca-1⁺*c-kit*⁺AA4.1⁺Lin⁻ cells), fetal liver progenitors and mature blood cells (AA4.1⁺), and adult bone marrow HSC (Rh-123^{low}Sca-1⁺*c-kit*⁺Lin⁻ cells), and (b) semiquantitative reverse transcription PCR (RT-PCR) using first strand cDNAs prepared from sorted Lin⁻Sca-1⁺ and Lin⁻Sca-1⁻ bone marrow cells according to the manufacturer's protocol (Clontech). Sca-1⁺*c-kit*⁺AA4.1⁺Lin⁻, AA4.1⁺, and Rh-123^{low}Sca-1⁺*c-kit*⁺Lin⁻ cell-specific cDNA libraries (prepared by Clontech's CapFinder cDNA library construction method) were a kind gift from Dr. Ihor Lemischka (Princeton University).

[0052] Both PCR and RT-PCRs were performed in duplex using different dilutions of cDNA libraries and first strand cDNAs, with mouse *Hepp* primers amplifying a 446-bp fragment (5' oligo 5'-CGAAGGAGTGGCGGGGTCTG-3' [SEQ ID NO: 8]; 3' oligo 5'-TTCCTTTGCCCTCGTGCTGA-3' [SEQ ID NO: 9]), and primers for hypoxanthine– guanine– phosphorybosyltransferase (HPRT) (5' oligo 5'-GTTGAGAGATCATCTCCACC-3' [SEQ ID NO: 10]; 3' oligo 5'-AGCGATGATGAACCAGGTTA-3' [SEQ ID NO: 11]) which amplify a 340-bp fragment as an internal positive control. Reactions were performed in an Eppendorf Mastercycler for 25–40 cycles (95°C for 30 s, 57°C for 45 s, 72°C for 30 s). *Hepp* expression in various hematopoietic lineages was also assessed by semiquantitative duplex RT-PCR. A panel of the following lineagespecific mouse hematopoietic cell lines was used: LyD9 (pluripotent progenitor cell line), FDC-P1 (myeloid progenitor cell line), 1881 (pro-B cell line), BaF/3 and 70Z/3 (pre-B cell lines), CH33 and M12 (B cell lines), NFS-70 (pro-B cell lymphoma), NFS-5 (pre-B cell lymphoma), A20 and WEHI-279 (B cell lymphoma lines), J558 (B cell myeloma), EL4 and WEHI 7.1 (T cell lymphoma), WEHI-3B (myelomonocytic cell line), and RAW 309 and J774A.1 (monocyte–macrophage cell lines). These cell lines can be obtained from the American Type Culture Collection (ATCC) Manassas, VA 20108. Total RNA (2 µg) from each cell line was reverse transcribed using random hexamers (Pharmacia, Piscataway, NJ) and MMLV reverse transcriptase (GIBCO) in a 20-µl reaction, and 2 µl of the first-strand cDNA was used as a template in a duplex PCR (30 cycles; 95°C for 30 s, 57°C for 45 s, 72°C for 30 s) with primers for *Hepp* and HPRT.

RESULTS

Isolation and Analysis of Full-Length cDNA for Hepp

[0053] After differential screening of subtracted Lin⁺Sca-1⁺ and Lin⁺Sca-1⁻ cell-specific libraries, differentially expressed cDNA clones were subjected to automated sequencing and computer assisted analysis. BLAST search of the GenBank/EMBL database identified one of the ESTs (LS215), isolated from Lin⁺Sca-1⁻ cell-specific subtracted cDNA library, as a novel gene. Based on the preferential expression in adult bone marrow progenitors the gene was designated *Hepp* for hematopoietic progenitor protein. Mouse cDNA clone for *Hepp* was isolated by PCR-based screening of arrayed full-length Lin⁺Sca-1⁻ cell specific cDNA library using sequence-specific primers. The two longest isolated clones were sequenced and analyzed. Mouse *Hepp* transcript (2082 bp) contains an open reading frame (ORF) of 711 bp with one in-frame stop codon upstream of the first ATG codon, and encodes a protein of 237 amino acids (theoretical Mr 26.1 kDa) with no known domains or motifs (Accession No. AF322238) (Fig. 1A). In the UniGene database mouse *Hepp* cDNA is represented by one cluster of uncharacterized ESTs (Mm.28595). Search of the human EST division of the GenBank database with the mouse *Hepp* cDNA sequence identified several homologous ESTs, that are identical to human FLJ20764 cDNA (Accession No. AK000771) of unknown function. FLJ20764 cDNA (1918 bp) contains partial ORF (609 bp) that encodes a 202 amino acid protein similar to mouse *Hepp* protein and is represented by one cluster of uncharacterized ESTs Hs.34045) in the UniGene database. According to the NCBI HomoloGene (www.ncbi.nlm.nih.gov/HomoloGene/), a homology resource which includes both curated and calculated orthologs and homologs for human, mouse, rat, zebrafish, cow and fly genes represented in the UniGene), mouse ESTs from UniGene cluster Mm.28595 and human hypothetical protein FLJ20764, represented by the UniGene cluster Hs.34045, are calculated orthologs with 88% sequence identity. All human ESTs from cluster Hs.34045 were assembled into a single contig with EST Assembly Machine (<http://gcg.tigem.it/cgi-bin/uniestass.pl>), conceptually translated in all six frames (<http://dot.imgen.bcm.tmc.edu:9331/seq-util/seq-util.html>) and compared with nucleotide and amino acid sequence of mouse *Hepp* and human FLJ20764 cDNA. Electronically extended cDNA (2082 bp) for human FLJ20764 contains an ORF of 723 bp with one in-frame stop codon upstream of the first ATG codon and encodes a 241 amino acid protein (theoretical Mr 26.1 kDa) (Accession No. AF322239) (Fig. 1B). ClustalW amino acid sequence alignment (32) has shown

that mouse *Hepp* and human FLJ20764 proteins share 73% identity and 77% similarity, with several highly conserved contiguous blocks of amino acids (Fig. 2), again indicating that FLJ20764 gene most likely represents the human ortholog of the mouse *Hepp* gene. Based on SMART analysis (Simple Modular Architecture Research Tool, <http://smart.emblheidelberg.de/>) (28), SwissProt database search, and search of the Conserved Domain Database using RPS-BLAST (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>), both mouse and human *Hepp* proteins lack any known domains or motifs, and do not have any obvious homology or structural similarities to known proteins. SignalP V1.1 Server (<http://www.cbs.dtu.dk/services/SignalP/>) did not predict the presence of N-terminal signal peptide or signal peptide cleavage sites in mouse and human *Hepp* protein. NetOGlyc 2.0 Server (<http://www.cbs.dtu.dk/services/NetOGlyc/>) has predicted one putative mucin type O-glycosylation site in mouse *Hepp* protein (Thr 213) and three putative O-glycosylation sites in human *HEPP* protein (Thr 81, 122, 217). NetPhos 2.0 protein phosphorylation prediction server (<http://www.cbs.dtu.dk/services/NetPhos/>), which predicts for serine, threonine, and tyrosine phosphorylation sites in eukaryotic proteins, has found 14 putative phosphorylation sites both in the mouse *Hepp* (Ser: 11; Thr: 2; Tyr: 1) and the human *HEPP* protein (Ser: 11; Thr: 2; Tyr: 1) (data not shown).

Identification and Analysis of Invertebrate and Vertebrate Orthologs of Hepp

[0054] Using the Proteome WormPD database (<http://www.proteome.com/databases>), DRES (*Drosophila* related expressed sequences) Search Engine (27) (<http://hercules.tigem.it/DRES/dres.html>) and *Drosophila* Genome Project Blast Search (http://www.fruitfly.org/cgi-bin/blast/public_blast.pl) we were not able to identify a *C. elegans* or *Drosophila* ortholog of *Hepp* gene. By screening the Gen-Bank nonredundant database with mouse cDNA we have identified several rat (UniGene cluster Rn.16249) and one zebrafish EST (Accession No. AW422282), similar to *Hepp* gene. All rat ESTs in cluster Rn.16249 represent the 3' untranslated region (3' UTR) of rat *Hepp* cDNA and thus could not be conceptually translated and compared with mouse and human *HEPP* proteins. However, at the nucleotide sequence level 3' UTR of rat *Hepp* cDNA shares 88 and 86% identity with mouse and human *HEPP* cDNAs, respectively (data not shown). The zebrafish EST, representing partial cDNA, was conceptually translated, analyzed with SMART, and compared

with protein sequence of mouse and human *HEPP*. ClustalW amino acid sequence alignment has shown that partial zebrafish *Hepp* protein shares 64% identity and 74% similarity with mouse *Hepp* protein, and 66% identity and 76% similarity with human *HEPP* protein (Fig. 3). The alignment of the N-terminal part of the zebrafish, mouse and human *HEPP* proteins demonstrates a high degree of evolutionary conservation of the amino terminal part of the protein and again shows several highly conserved contiguous blocks of amino acids (Fig. 3).

Expression Analysis of Hepp

[0055] Hybridization of mouse multiple tissue Northern blot has revealed that *Hepp* is expressed at a very low level in the heart, lung, spleen and thymus, and at a higher level in the muscle. The heart and muscle express a larger ~4.8-kb transcript, whereas lung, spleen, and thymus express a smaller ~4-kb isoform, which probably arises through alternative splicing. *Hepp* transcripts are not detectable in the brain, kidney, liver, skin, intestine, stomach, and testis (Fig. 4). Since *Hepp* was found to be expressed preferentially in a progenitor cell population after the differential screening of subtracted $\text{Lin}^- \text{Sca-1}^+$ and $\text{Lin}^- \text{Sca-1}^-$ cell-specific libraries, it was important to reanalyze its expression in populations of mouse fetal and adult HSC and progenitors. Repetitive semi-quantitative duplex PCR analysis (using various dilutions of cDNA libraries as the template and 25–40 PCR cycles) has shown that *Hepp* is not expressed in mouse fetal liver HSC ($\text{Sca-1}^+ c\text{-kit}^+ \text{AA4.1}^+ \text{Lin}^-$ cells), but is highly expressed in progenitor cell population (AA4.1^- cells) (Fig. 5). Similarly, using semi-quantitative duplex PCR with various dilutions of cDNA library and 25–40 PCR cycles, *Hepp* transcript was not detectable in highly purified population of $\text{Rh-123}^{\text{low}} \text{Sca-1}^+ c\text{-kit}^+ \text{Lin}^-$ bone marrow cells. This population represents ~0.001% of normal bone marrow cells and is highly enriched for HSC activity (17, 29). Interestingly, expression of *Hepp* was found to be upregulated as more heterogeneous population of HSC and progenitors ($\text{Lin}^- \text{Sca-1}^+$ cells, representing 0.1–0.2% of normal bone marrow cells) differentiates into progenitors ($\text{Lin}^- \text{Sca-1}^-$ cells), as analyzed by semiquantitative duplex RT-PCR (Fig. 5). These findings confirm the results of differential screening of $\text{Lin}^- \text{Sca-1}^+$ and $\text{Lin}^- \text{Sca-1}^-$ cell-specific subtracted libraries. RT-PCR analysis of various hematopoietic cell lines has shown that *Hepp* is ubiquitously expressed in lymphoid progenitor, pro-B, pre-B and B cell lines including lymphomas, in T cell lymphoma cell lines and thymus, and in myeloid progenitor and monocyte–macrophage cell lines (Fig. 6).

Human HEPP maps to chromosomal region with frequent chromosome aberrations associated with multiple cases of various hematological malignancies

[0056] Using the CELERA Gene Discovery System and BAC mapping it was determined that mouse *Hepp* gene maps to telomeric part of the chromosome 12, whereas human *HEPP* gene maps to q32 region on human chromosome 14, depicted in Figure 7. According to Breakpoint Map of Recurrent Chromosome Aberrations database (<http://www.ncbi.nlm.nih.gov/CCAP/mitelsum.cgi>), band 14q32 represents a region with frequent balanced (translocations) and unbalanced chromosome aberrations (deletions, duplications) associated with multiple cases of various hematological malignancies (Table 1), for some of which the genes involved are unknown.

Table 1

Neoplasm	Cases
Acute myelomonocytic leukemia	5
Lymphoblastic lymphoma	13
Chronic lymphocytic leukemia	185
Acute lymphoblastic leukemia	316
Multiple myeloma	190
B-prolymphocytic leukemia	24
Plasma cell leukemia	35
Adult T-cell lymphoma/leukemia	31
Diffuse large B-cell lymphoma	324
Nodal marginal zone B-cell lymphoma	2
Burkitt's lymphoma	127
Follicular lymphoma	515
Hairy cell leukemia	8
Mantle cell lymphoma	158
Splenic marginal zone B-cell lymphoma	21
T-prolymphocytic leukemia	51

Mapping of the human *HEPP* to a chromosomal region with frequent chromosome aberrations associated with multiple cases of various hematological malignancies, suggests that *HEPP* is involved in etiology of some of the hematological malignancies.

Mouse Hepp is expressed in almost all parts of central and peripheral nervous system throughout embryonic development and in adult mice, and is expressed in neural stem cells and progenitors

[0057] Expression of *Hepp* was analyzed in mouse fetal neural stem cells (NSC) and progenitors. Cultures of NSC and progenitors from E14 embryonic brain subventricular zone (SV) were established in the presence of bFGF (basic Fibroblast Growth Factor or FGF-2, necessary for NSC maintenance) and were induced to differentiate into neurons and glial cells by withdrawal of bFGF or by addition of ciliary neurotrophic factor (CNTF), platelet derived growth factor (PDGF- β) or bone morphogenic protein 7 (BMP7), cytokines used to drive differentiation of NSC in to neurons and glia cells in culture. Cyclophilin A was used as an internal positive control in RT-PCR. These experiments have established that *Hepp* is ubiquitously expressed in fetal NSC, progenitors and differentiated neural cell types (Fig. 8).

[0058] Using Mouse Brain Rapid-Scan Panel (OriGene Technologies), the expression pattern of *Hepp* in embryonic and adult central and peripheral nervous system was analyzed. The results demonstrated that *Hepp* is expressed in almost all parts of central and peripheral nervous system throughout embryonic development and in adult mice (including forebrain, midbrain, hindbrain, spinal cord) (Fig. 9).

Generation of *Hepp* knockout mice

[0059] Searching the database of trapped genes (Dr. William Skarnes, UC Berkeley) (<http://socratesberkeley.edu/~starnes/resource.html>), we identified ES clone KST303 in which allele for HEPP was trapped by ATG-less secretory gene trap vector pGT1.8TM β geo. The gene trap vector pGT1.8TM β geo contains a splice acceptor sequence and transmembrane protein domain TM of CD4 gene upstream of a reporter and is activated following insertion into an intron. The analysis of trapping event in ES cell clone KST303 showed proper splicing of the integrated vector and fusion of the β geo reporter to the 5' UTR of HEPP transcript, which should result in severely truncated transcript and absence of functional HEPP protein. Using ES cell clone KST303 we generated HEPP knockout mice. ES clones with targeted *Hepp* alleles can be generated by routine means by a practitioner skilled in gene targeting techniques. (See, for

example, Gene Knockout Protocols. In: Methods in Molecular Biology, vol. 158, 2001. Edited by: M.J. Tymms and I. Kola. Humana Press, Totowa, New Jersey.)

[0060] Viable heterozygous *Hepp* mice were bred to generate *Hepp*^{-/-} mice (Fig. 10).

Genotyping of progeny from breeding of *Hepp*^{+/-} mice has revealed that the vast majority (80%) of *Hepp*^{-/-} mice die *in utero* (Fig. 10; Table 2).

Table 2. Genotyping and ratio of adult *Hepp*^{+/+}, *Hepp*^{+/-} and *Hepp*^{-/-} mice.

Genotype →	<i>Hepp</i> ^{+/+}	<i>Hepp</i> ^{+/-}	<i>Hepp</i> ^{-/-}	Total
Number of mice	15	34	3	52
Experimental Mendelian ratio	23.5%	53%	4.7%	
Theoretical Mendelian ratio	16 (25%) 1	32 (50%) 2	16 (25%) 1	64

Analysis of hematopoietic system in *Hepp* KO mice

[0061] The analysis of 23 *Hepp*^{+/-} mice revealed perturbed hematopoiesis consisting of bone marrow cytopenia, overproduction and/or accumulation of hematopoietic progenitors, and splenomegaly with follicular hyperplasia. In addition, *Hepp*^{+/-} mice have significantly reduced number of bone marrow (BM) cells in femurs and tibias (Fig. 11).

[0062] Flow cytometry analysis revealed decreased content of B cells, granulocytes, macrophages and erythroblasts in the BM of *Hepp*^{+/-} mice (Fig. 12). In contrast, the content of BM cell populations containing (a) immature hematopoietic cells (lineage negative Lin⁻ cells), (b) early and late progenitors (Lin⁻Sca-1⁻ and Lin⁻c-kit⁻ cells), and (c) early progenitors and HSC (Lin⁻Sca-1⁺ and Lin⁻c-kit⁺ cells) was increased in *Hepp*^{+/-} mice (Fig. 13). Furthermore, colony-forming assays demonstrated increased content of blast colony-forming (CFU-Blast), myelo-erythroid (CFU-GM, BFU-E and CFU-Meg) and lymphoid (CFU-B) progenitors in the BM from *Hepp*^{+/-} mice (Fig. 14).

[0063] Another readily observable feature was very frequent splenomegaly in *Hepp*^{+/-} mice, with significantly increased number of splenocytes and follicular hyperplasia (Fig. 15). This

follicular hyperplasia was accompanied by increased content of B220⁺ cells in the spleen of *Hepp*^{+/-} mice as analyzed by flow cytometry (Fig. 16). Flow cytometry analysis of myeloid cells (granulocytes, macrophages and erythroblasts) in the spleen did not show any difference between wild type and *Hepp*^{+/-} mice (data not shown). We have also observed slight decrease in the content of CD4⁺ T cells and significantly decreased content of CD8⁺ T cells in the spleen of *Hepp*^{+/-} mice as analyzed by flow cytometry (Fig. 17).

Analysis of central and peripheral nervous system in *Hepp* KO mice

[0064] The last facet of the phenotype is progressive neuromuscular degeneration in *Hepp*^{+/-} mice. About 40% of *Hepp*^{+/-} mice show slight tremor, impaired balance during walking, and very mild paralysis of hind legs. Mice have difficulty turning over when placed on their backs in a supine position.

[0065] After 4 months of age about 10% of affected *Hepp*^{+/-} mice exhibit full paralysis of hind legs, seizures, severe muscular atrophy and wasting (Fig. 18). Mice with full penetrance of the progressive neurodegenerative disease do not survive beyond 6 months of age. A review of current literature and mouse models (e.g. mice lacking hypoxia-response element of VEGF; Oosthuyse B, Moons L, Storkebaum E, et al. (2001). Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. Nat. Genet. 2001 Jun;28(2):131-138), supports the conclusion that adult-onset progressive neurodegenerative disease in *Hepp*^{+/-} mice has features that closely resemble amyotrophic lateral sclerosis (ALS). Accordingly, these mice show promise of being a useful model for the study of this human disease.

CONCLUSIONS

[0066] In summary, the multifaceted phenotype of *Hepp*^{+/-} mice consists of at least the following features:

[0067] 1. Skeletal defects and growth retardation, indicating that *Hepp* plays a role in embryonic development,

[0068] 2. Perturbed hematopoiesis that encompasses: bone marrow cytopenia, overproduction and accumulation of hematopoietic progenitors, and splenomegaly with follicular hyperplasia, and

[0069] 3. Adult-onset progressive neurodegenerative disease reminiscent of amyotrophic lateral sclerosis (ALS), which suggests a role for *Hepp* in neuronal development and function.

[0070] The complex phenotype of *Hepp* KO mice suggests that *Hepp* is a part of common molecular mechanism utilized in the development and differentiation of hematopoietic and neuronal cells and perhaps other cell types as well.

DISCUSSION

[0071] Differential screening of subtracted cDNA libraries from mouse fetal and adult cell populations enriched for HSC and progenitors and sequencing of differentially expressed clones have already yielded a number of both novel as well as evolutionarily conserved genes, present from *Drosophila* to humans (16, 17, 19, 31). Described herein is the cloning and characterization of a novel gene, *Hepp*, identified through differential screening of subtracted cDNA libraries from mouse adult bone marrow cell populations enriched for HSC (Lin⁻Sca-1⁺ cells) and progenitors (Lin⁻Sca-1⁻ cells) (19). Mouse *Hepp* and human *HEPP* transcripts encode novel conserved proteins without any known structural or functional domains or motifs, and lacking any obvious homology or structural similarities to known proteins. Furthermore, lack of invertebrate orthologs and a high degree of evolutionary conservation of the peptide sequence in zebrafish, mouse and humans suggest that in vertebrates *Hepp* gene has an important conserved although as yet not completely elucidated function. Differential screening of mouse bone marrow HSC (Lin⁻Sca-1⁺) and progenitor (Lin⁻Sca-1⁻) cell-specific subtracted cDNA libraries has demonstrated that *Hepp* is expressed preferentially in progenitor cell populations (Lin⁻Sca-1⁻ cells). During embryonic blood cell development *Hepp* is not expressed in the population of mouse fetal liver HSC (Sca-1⁺*c-kit*⁺AA4.1⁺Lin⁻ cells), but is abundantly transcribed in fetal liver progenitors and mature blood cells (AA4.1⁻ cells). These results are in agreement with the fact

that in the BLAST search of the Stem Cell Database (SCDB; <http://stemcell.princeton.edu/>; Dr. Ihor Lemischka, Princeton University) mouse *Hepp* cDNA did not match any ESTs derived from the Sca-1⁺*c-kit*⁺AA4.1⁺Lin⁻ cell-specific subtracted library, containing transcripts expressed preferentially in mouse fetal liver HSC population (17, 30).

[0072] Similarly, during adult mouse hematopoiesis, *Hepp* is not transcribed in the population of Rho-123^{low}Sca-1⁺*c-kit*⁺Lin⁻ cells (representing ~0.001% of normal bone marrow cells and highly enriched for HSC) (17, 29), but is expressed at low level in more heterogeneous population of Lin⁻Sca-1⁺ cells (representing 0.1–0.2% of normal bone marrow cells and enriched for HSC and progenitors). *Hepp* transcription is upregulated in progenitor cell population (Lin⁻Sca-1⁻ cells) and in various lymphoid and myeloid cell lines. Therefore, mouse *Hepp* exhibits developmentally regulated pattern and conservation of preferential expression in both fetal and adult hematopoietic progenitors and mature blood cells during embryonic and adult hematopoiesis. Restricted expression pattern in tissues and preferential expression in mouse fetal and adult hematopoietic progenitors and mature blood cells suggest that mouse *Hepp* is involved in the regulation of HSC and progenitor cell lineage commitment and differentiation.

[0073] In describing preferred embodiments of the present invention, specific terminology is employed for the sake of clarity. However, the invention is not intended to be limited to the specific terminology so selected. It is to be understood that each specific element includes all technical equivalents, which operate in a similar manner to accomplish a similar purpose. Each reference cited herein is incorporated by reference as if each were individually incorporated by reference.

[0074] The embodiments illustrated and discussed in the present specification are intended only to teach those skilled in the art the best way known to the inventors to make and use the invention, and should not be considered as limiting the scope of the present invention. The exemplified embodiments of the invention may be modified or varied, and elements added or omitted, without departing from the invention, as appreciated by those skilled in the art in light of the above teachings. It is therefore to be understood that, within the scope of the claims and their equivalents, the invention may be practiced otherwise than as specifically described.

REFERENCES

References cited herein are listed below for convenience and are hereby incorporated by reference.

1. Orkin, S. H. (1996) Development of the hematopoietic system. *Curr. Opin. Genet. Dev.* **6**, 597–602.
2. Morrison, S. J., Wright, D. E., Cheshier, S. H., and Weissman, I. L. (1997) Hematopoietic stem cells: Challenges to expectations. *Curr. Opin. Immunol.* **9**, 216–221.
3. Morrison, S. J., Shah, N. M., and Anderson, D. J. (1997) Regulatory mechanisms in stem cell biology. *Cell* **88**, 287–298.
4. Kondo, M., Weissman, I. L., and Akashi, K. (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91**, 661–672.
5. Akashi, K., Traver, D., Miyamoto, T., and Weissman, I. L. (2000) A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* **404**, 193–197.
6. Weiss, M. J., and Orkin, S. H. (1995) GATA transcription factors: Key regulators of hematopoiesis. *Exp. Hematol.* **23**, 99–107.
7. Watowich, S. S., Wu, H., Socolovsky, M., Klingmuller, U., Constantinescu, S. N., and Lodish, H. F. (1996) Cytokine receptor signal transduction and the control of hematopoietic cell development. *Annu. Rev. Cell Dev. Biol.* **12**, 91–128.
8. Tenen, D. G., Hromas, R., Licht, J. D., and Zhang, D. E. (1997) Transcription factors, normal myeloid development, and leukemia. *Blood* **90**, 489–519.
9. Glimcher, L. H., and Singh, H. (1999) Transcription factors in lymphocyte development—T and B cells get together. *Cell* **96**, 13–23.
10. Rothenberg, E. V. (1999) Stepwise specification of lymphocyte developmental lineages. *Curr. Opin. Genet. Dev.* **10**, 370–379.
11. Kuo, C. T., and Leiden, J. M. (1999) Transcriptional regulation of T lymphocyte development and function. *Annu. Rev. Immunol.* **17**, 149–187.
12. Cortes, M., Wong, E., Koipally, J., and Georgopoulos, K. (1999) Control of lymphocyte development by the Ikaros gene family. *Curr. Opin. Immunol.* **11**, 167–171.

13. Busslinger, M., Nutt, S. L., and Rolink, A. G. (2000) Lineage commitment in lymphopoiesis. *Curr. Opin. Immunol.* **12**, 151–158.
14. O’Riordan, M., and Grosschedl, R. (2000) Transcriptional regulation of early B-lymphocyte differentiation. *Immunol. Rev.* **175**, 94–103.
15. Kondo, M., Scherer, D. C., Miyamoto, T., King, A. G., Akashi, K., Sugamura, K., and Weissman, I. L. (2000) Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines. *Nature* **407**, 383–386.
16. Lemischka, I. (1999) Searching for stem cell regulatory molecules. *Ann. N.Y. Acad. Sci.* **872**, 274–287.
17. Phillips, R. L., Ernst, R. E., Brunk, B., Ivanova, N., Mahan, M. A., Deanehan, J. K., Moore, K. A., Overton, G. C., and Lemischka, I. R. (2000) The genetic program of hematopoietic stem cells. *Science* **288**, 1635–1640.
18. Weissman, I. L. (2000) Translating stem and progenitor cell biology to the clinic: Barriers and opportunities. *Science* **287**, 1442–1446.
19. Abdullah, J. M., Li, X., Nachtman, R. G., and Jurecic, R. (2001) FLRF, a novel evolutionarily conserved ring finger gene, is differentially expressed in mouse fetal and adult hematopoietic stem cells and progenitors. *Blood Cells Mol. Dis.* **27**, 320–333.
20. Spangrude, G. J., and Scollay, R. (1990) A simplified method for enrichment of mouse hematopoietic stem cells. *Exp. Hematol.* **18**, 920–926.
21. Jurecic, R., Van, N. T., and Belmont, J. W. (1993) Enrichment and functional characterization of Sca-1⁺WGA⁺, Lin⁺WGA⁺, Lin⁺Sca-1⁺, and Lin⁺Sca-1⁺WGA⁺ bone marrow cells from mice with an Ly-6a haplotype. *Blood* **82**, 2673–2683.
22. Rebel, V. I., Dragowska, W., Eaves, C. J., Humphries, R. K., and Lansdorp, P. M. (1994) Amplification of Sca-1⁺ Lin⁺ WGA⁺ cells in serum-free cultures containing steel factor, interleukin-6, and erythropoietin with maintenance of cells with long-term *in vivo* reconstituting potential. *Blood* **83**, 128–136.
23. Li, C. L., and Johnson, G. R. (1995) Murine hematopoietic stem and progenitor cells: I. Enrichment and biologic characterization. *Blood* **85**, 1472–1479.
24. Munroe, D. J., Loebbert, R., Bric, E., Whitton, T., Prawitt, D., Vu, D., Buckler, A., Winterpacht, A., Zabel, B., and Housman, D. E. (1995) Systematic screening of an arrayed cDNA library by PCR. *Proc. Natl. Acad. Sci. USA* **92**, 2209–2213.

25. Diatchenko, L., Lau, Y. F., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E. D., and Siebert, P. D. (1996) Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA* **93**, 6025–6030.
26. Diatchenko, L., Lukyanov, S., Lau, Y. F., and Siebert, P. D. (1999) Suppression subtractive hybridization: A versatile method for identifying differentially expressed genes. *Methods Enzymol.* **303**, 349–380.
27. Banfi, S., Borsani, G., Rossi, E., Bernard, L., Guffanti, A., Rubboli, F., Marchitello, A., Giglio, S., Coluccia, E., Zollo, M., Zuffardi, O., and Ballabio, A. (1996) Identification and mapping of human cDNAs homologous to *Drosophila* mutant genes through EST database searching. *Nat. Genet.* **13**, 167–174.
28. Schultz, J., Copley, R. R., Doerks, T., Ponting, C. P., and Bork, P. (2000) SMART: A Web-based tool for the study of genetically mobile domains. *Nucleic Acids Res.* **28**, 231–234.
29. Kim, M., Cooper, D. D., Hayes, S. F., and Spangrude, G. J. (1998) Rhodamine-123 staining in hematopoietic stem cells of young mice indicates mitochondrial activation rather than dye efflux. *Blood* **91**, 4106–4117.
30. Jordan, C. T., Astle, C. M., Zawadzki, J., Mackarechtschian, K., Lemischka, I. R., and Harrison, D. E. (1995) Long-term repopulating abilities of enriched fetal liver stem cells measured by competitive repopulation. *Exp. Hematol.* **23**, 1011–1015.
31. Wiesmann, A., Phillips, R. L., Mojica, M., Pierce, L. J., Searles, A. E., Spangrude, G. J., and Lemischka, I. (2000) Expression of CD27 on murine hematopoietic stem and progenitor cells. *Immunity* **12**, 193–199.
32. Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673–4680).